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ROLE OF WORMWOOD (*ARTEMISIA ABSINTHIUM*) EXTRACT ON OXIDATIVE STRESS IN AMELIORATING LEAD INDUCED HAEMATOTOXICITY.

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Abstract

Effects of ROS generation have been postulated to be major contributors to lead-exposure related disease. The aim of the study was to investigate the effect of aqueous extract of wormwood (*Artemisia absinthium*) on oxidative stress in rats protractedly exposed to lead. Aqueous extract of wormwood plant was administered orally (200 mg.kg^{-1} body weight). Plasma vitamin C, E and non-protein thiol concentrations, red blood cells (RBC) thiobarbituric acid reactive substances, reduced glutathione levels and haemolysis test were evaluated. In addition, RBC antioxidant enzymes activities such as superoxide dismutases, catalase, glutathione peroxidase, glutathione reductase were also estimated. After 11-weeks, significant decreases of plasma vitamin C, E, non protein-thiol (NP-SH) and RBC-reduced glutathione levels were observed in Pb compared to control group (-32.9%, -57.1%, -53.1%, -33.9%, respectively); superoxide dismutase, glutathione peroxidase, uric aminolevulinic acid and haemolysis test significantly increased in Pb compared to control group (+64.3%, +40.3%, +145%, +44.3%, respectively). In our investigation, after 4-weeks of treatment all treated groups did not show any difference compared to the control group, except for glutathione peroxidase and RBC-superoxide dismutase activity (-15.7% and +16.4%, respectively). The findings of this study suggest that wormwood (*Artemisia absinthium*) extract restored the enzymes activities perturbed by exposure to lead.
and had a protective role against lipid peroxidation

Key words: Antioxidant defence system - Lead acetate - Lipid peroxidation - Red Blood Cell – Rat - Wormwood (*Artemisia absinthium*) plant extract.

AbbreviationS: ROS, reactive oxygen species; Pb, lead acetate; GSH, reduced glutathione; GSSG, glutathione disulfide; SOD, superoxide dismutase; GPx, glutathione peroxidase; TBARS, Thiobarbituric acid reactive substances.

Introduction

The toxicokinetics of lead is a complex process (Leggett, 1993; Cory-Schlecta and Schaumburg, 2000). The primary routes of lead absorption are via respiration and ingestion; cutaneous absorption is negligible. Absorbed lead is cleared by the kidneys in the urine and unabsorbed lead is eliminated in the faeces. Absorbed lead is carried in the blood circulation, wherein the major burden (95%) is on the erythrocytes and partly to other tissues-in the plasma. In addition to killing cells via excitotoxicity and apoptosis, lead also causes toxic effects by oxidative stress

either directly or by indirectly-produced lipid peroxidation. Lead alters lipid metabolism, enhances lipid peroxidation and decreases cell membrane fluidity of developing rats (Gurer and Ercal; 2000; Villeda-Hernandez et al., 2001). Additionally to membrane peroxidation, lead exposure causes haemoglobin oxidation, which can also cause RBC haemolysis. The mechanism responsible for this reaction is lead-induced inhibition of delta aminolevulinic acid dehydrogenase (ALAD). As a result, elevated levels of the substrate ALA are found in both the blood and urine of lead-exposed subjects (Farant and Wigfield; 1982). Elevated levels of ALA generate hydrogen peroxide (H_2O_2) and superoxide radical (O_2^-), and also interact with oxyhemoglobin, resulting in the generation of hydroxyl radicals (OH), the most reactive of the free radicals (Bechara, 1996; Courtois et al., 2003).

Antioxidant enzymes and glutathione play an important role in the defence against ROS. Most experimental research has indicated that after exposure to lead there is an increased amount of oxidized glutathione (GSSG) with a parallel drop of reduced glutathione (GSH) in different organs (Gurer et al., 1999; Sivaprasad et al., 2003). Similarly, incubation of human blood in the presence of lead compounds causes a drop of GSH concentration (Hunaiti and Soud, 2000). Glutathione is necessary for proper functioning of glutathione peroxidase (GPx) and glutathione-S-transferase (GST) enzymes taking part in the elimination of noxious compounds and ROS. Several researches have shown that the above-mentioned enzymes, together with glutathione reductase (GR), may be modified under the influence of lead (Hunaiti and Soud, 2000, Gurer et al., 1998). There are both superoxide dismutase (SOD) isoenzymes mitochondrial and cytosol in blood plasma, whereas cytosol isoenzyme is found only in mature erythrocytes (lacking mitochondria). The biological role of SOD is to dismute superoxide ion; hydrogen peroxide (H_2O_2) produced in this reaction is eliminated by catalase (CAT), one of the most active enzymes in the human organism. CAT consists of 4 protein subunits, each of them containing haem group with iron (Fe), connected to its active centre. The highest activity of CAT in the human body may be found in the liver and red blood cells (Aebi, 1984; Gaetani et al. 1994).

Wormwood extract have high contents of total phenolic compounds and total flavonoids indicating that these compounds contribute to antiradical and antioxidative activity (Jasna et al. 2004). Thus, the aim of this study was to evaluate the effect of the aqueous extract of Wormwood on the antioxidant status and its protective ability against oxygen reactive species in rats chronically exposed to lead.

Materiels and Methods

Preparation of wormwood (*Artemisia absinthium*) plant extracts (Abs):

Whole plants of *A. absinthium* (voucher No. 0006) were collected from Mecheria, in west district of Algeria in the month of May. The plant was identified and authenticated at the Herbarium of Botany Directorate in Es-senia (Oran) University. 500 gm of whole wormwood plants were extracted with 1.5 L of distilled water by the method of continuous hot extraction at 60°C for 60 mins. This procedure was repeated, and the filtrate was lyophilized. The residue collected (yield 75 g) was stored at -20°C.

Animals and Experimental design

In the experiment, 30 male Wistars rats (40 ± 6g, 18 intoxicated rats, and 12 normal rats) were used. The rats were housed five per cage and had free access to food and water. They were exposed to a 10-14 h light-dark cycle and the room temperature was controlled at 23 ± 2°C. Animals were first exposed to Pb at the age of 2-3 weeks, when they were weighed. Experiments were performed on rats aged 11 weeks: 1) Pb group: - Rats exposed to Pb (750 mg/kg, in the form of Pb acetate in their drinking water *ad libitum*) for 11-weeks. 2) Control group: - Rats that received water for 11-weeks. 3) Pb/water group: - Rats intoxicated by Pb for 11-weeks, receiving water for 4 additional weeks. 4) Pb/ Abs groups: - Rats treated with Pb receive aqueous wormwood extract (Abs) (200 mg/kg, in their drinking water *ad libitum*) during 4-weeks. 5) Water/Abs group - Group untreated with lead received aqueous wormwood extract for 4 additional weeks. Blood samples were collected at the end of 11-weeks of intoxication and 4-weeks of treatment, and all the rats were killed by decapitation after Pentobarbitone sodium anesthesia (60 mg/kg). The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in the European Community guidelines (EEC Directive of 1986; 86/609/EEC). Blood was collected in two tubes with EDTA. In one tube haemoglobin and lead measurement was carried out. The other tube was centrifuged at 2000 x g for 10 mins at 4°C. Plasma was collected carefully and the RBC was washed three times in 0.9 g/L Nail solution. Lysed RBC were prepared by putting cells through three freeze-thaw cycles in dry ice and by the addition of five volumes of ice-cold distilled water. Cell membranes were removed by centrifugation at 10000×g, and the supernatant was used for the assay of enzymes activity.

Biochemical analysis

Haemolysis test: Washed RBC with normal saline was made into a 0.5 % suspension. H_2O_2 (100 mmol/L) induced haemolysis was tested after 1 h incubation of RBC suspension at 37 °C with tested drugs as previously described (Wu et al., 1997). The absorbance at 415 nm of control tubes was defined as 100%. The extent of haemolysis was calculated by referring to control tube.

Estimation of lipid peroxidation and non-protein thiol*:* Lipid peroxidation in erythrocytes was estimated colorimetrically by thiobarbituric acid reactive substances TBARS by the method of Niehius and Samuelsson (1968). In brief, 0.1 ml of plasma and erythrocyte was treated with 2 ml of (1:1:1 ratio) TBA-TCA-HCl reagent (thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA) and placed in water bath for 15 min, and cooled. The absorbance of clear supernatant was measured against reference blank at 535 nm. The non protein-thiol (NP-SH) content was measured in plasma by the method of Ellman (1959). Briefly, plasma was treated with TCA solution (5% for plasma). The thiol content was determined in the supernatants by reaction with dithionitrobenzoic acid (DTNB). The values are expressed as mg/dl.

Assay of Catalase and Superoxide dismutase: CAT was assayed colorimetrically at 620 nm, in RBC and expressed as µmoles of H_2O_2 consumed/min/mg Hb as described by Sinha (1972). The reaction mixture (1.5 ml, vol) contained 1.0 ml of 0.01 M pH 7.0 phosphate buffer, 0.1 ml of erythrocytes supernatant and 0.4 ml of 2 M H_2O_2. The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio). SOD was assayed in erythrocyte supernatant utilizing the technique of Kakkar et al. (1984) based on inhibition of the formation of nicotinamide adenine dinucleotide, phenazine methosulfate and amino blue tetrazolium formazan. A single unit of enzyme was expressed as 50% inhibition of NBT (Nitroblue tetrazolium) reduction/min/mg Hb. The activity of GR in RBC supernatant was determined by measuring NADPH oxidation at 340 nm in the presence of oxidized glutathione (Glatzle et al., 1974) and expressed as *µ*moles of GSSH reduced/min/mg Hb.

Determination of Glutathione peroxidase and reduced glutathione: GPx activity was measured by the method described by Rotruck et al. (1973). Briefly, reaction mixture contained 0.2 ml of 0.4 M Tris-HCl buffer pH 7.0, 0.1 ml of 10 mM sodium azide, 0.2 ml of homogenate (homogenized in 0.4 M, Tris-HCl buffer, pH 7.0), 0.2 ml glutathione, and 0.1 ml of 0.2 mM hydrogen peroxide. The contents were incubated at 37°C for 10 min. The reaction was arrested by 0.4 ml of 10% TCA, and centrifuged. Supernatant was assayed for glutathione content by using Ellmans reagent (19.8 mg of 5, 5'-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1% sodium nitrate). The reduced glutathione level was determined using the method of Beutler and Kelly (1963). The technique involves protein precipitation by meta-phosphoric acid and a spectrophotometric assay at 412 nm of the yellow derivative obtained by the reaction of the supernatant with 5-5' dithiobis-2-nitrobenzoic acid. Glutathione peroxidase activity was expressed as µg of GSH consumed/min/mg Hb and reduced glutathione as mg/mg Hb.

Assay of Vitamin E and Vitamin C: The vitamin E level in the plasma was determined using the method of Desai (1984). Vitamin E in the lipid residue forms a pink colored complex with bathophenanthroline-orthophosphoric acid in the presence of ferric chloride. The absorption due to the pink complex was measured at 536 nm. The level of vitamin C in the plasma was determined using the method of Omaye et al. (1979). Vitamin C presents in the plasma forms a colored product on treatment with 2, 4-dinitrophenylhydrazine in the presence of copper sulfate. The absorbance of the colored product was measured at 520 nm.

Estimation of uric δ-aminolevulinic acid: Urine was collected for a three day period. Delta-aminolevulinic acid (ALA) was estimated in urine samples by the method of Grabecki (1967). Delta-aminolevulinic acid reacted with acetyl acetone and formed pyrrole substance which reacted with dimethylaminobenzoese aldehyde. The coloured complex was measured spectrophotometrically at 553 nm. The results were expressed as mg/dl.

Assay of lead metal in blood and urine: Pb concentration was determined in blood and urinate by atomic absorption spectrophotometry with a Zeeman-corrected graphite furnace (Model Spectra AA-220Z) and values were expressed in $\mu g.dl^{-1}$ in blood and $\mu g.day^{-1}$ in urine.

Haemoglobin estimation: The haemoglobin concentration was determined by the cyanmethemoglobin method Drabkin and Austin (1935).

Statistical analysis: All data were expressed as mean ± SEM of number of experiments (n = 6). The statistical significance was evaluated by one-way analysis of variance (ANOVA) using Student's "t" test to compare the differences between control and experimental groups. A value of $p < 0.05$ was considered to indicate a significant difference between groups.

Results

Statistically significant difference in blood and urine lead concentration was observed between Pb group at 11-weeks of intoxication compared to Pb/Abs and Pb/water groups (Table.1). The levels of uric δ-aminolevulinic were significantly lower in control vs. Pb groups and then the Pb/water groups; the values of uric δ-aminolevulinic are significativelly decreased in Pb/Abs group then the Pb group (-54.74%). The haemolysis test in Pb and Pb/water groups is significativelly increased compared to control group by +41%, +27%, Pb/Abs group indicate a significant reduction compared to the Pb group (-41%). The haemolysis test induced by H_2O_2, indicate that intoxication by lead increase the haemolytic effect, whereas after treatment with the Wormwood extract, it decreases the haemolyse of RBC (Water/Abs and PB/Abs) (Figure 1).

Figure 1 shows the levels of TBARS in the RBC of all groups. The level of TBARS was significantly increased in RBC of Pb and Pb/water groups compared to control group at 11-week. We have noticed a respectively +85.9%, and +95.2% elevation in the TBARS value in the RBC of Pb group and Pb/water group relative to the control group; but in

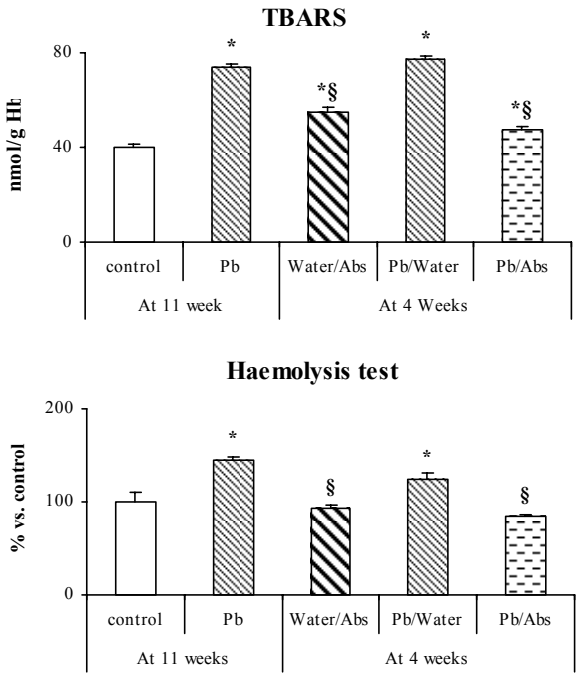

Figure. 1: TBARS level and haemolysis test value in all groups. The values expressed as mean ± SEM (n=6). all group vs. control group at 11-weeks, * P<0.05; and all group *vs.* Pb group at 11-weeks, § P<0.05 (Student's ' *t* ' test).

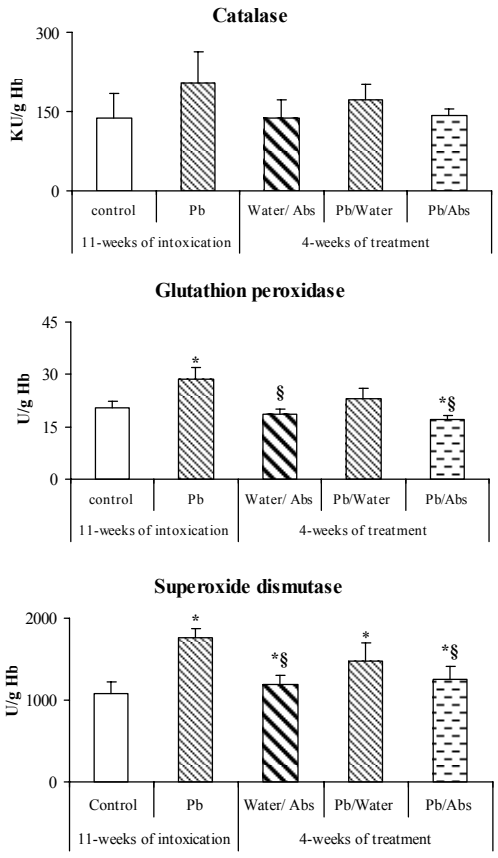

Figure 2: Catalase, Superoxide dismutase, glutathionperoxidase and glutathione reductase activity in RBC.The values expressed as mean ± SEM (n=6). all group vs. control group at 11-weeks, P<0.05; and all group *vs.* Pb group at 11-weeks, § P<0.05 (Student's ' *t* ' test).

Table 1. Blood (PbB), uric lead level (PbU) and Uric γ-aminolevulinic acid concentration in all groups.

	11-weeks of intoxication		4-weeks of treatment by plant extract		
	Control	Pb group	water/ Abs groups	Pb/water group	Pb/Abs groups
PbU µg.day^{-1}	0.05 ± 0.02	6.94 ± 1.7	0.10 ± 0.03§	2.11 ± 1.23*§	1.12 ± 1.48*§
PbB µg.dl^{-1}	0.15 ± 0.03	55.62 ± 6.30*	0.19 ± 0.02§	22.3 ± 5.78*§	15.29 ± 6.21*§
Uric γ-aminolevulinic acid mg.dl^{-1}	2.02 ± 0.35	4.95 ± 0.57*	2.45 ± 0.39§	3.20 ± 0.23*§	2.71 ± 0.67§

The values expressed as mean ± SEM (n=6). all group vs. control group at 11-weeks, * P<0.05; and all group *vs.* Pb group at 11-weeks, § P<0.05 (Student's ' *t* ' test).

Table 2. NP-SH, vitamin C and Vitamin E level in plasma and reduced GSH in RBC

	11-weeks of intoxication		4-weeks of treatment		
	control	Pb	water/Abs	Pb/water	Pb/Abs
NP-SH (mg.dl^{-1})	34,58 ± 2,42	16,22 ± 1,11*	33,55 ± 2,36§	21,10 ± 1,85*§	29,65 ± 2,37*§
GSH (µmol.L^{-1})	116.95 ± 12.55	70.07 ± 8.57*	96.48 ± 9.29§	78.65 ± 10.48*	118.59 ± 6.95§
Vitamin C (mg.dl^{-1})	2.31± 0.27	1.55 ± 0.13*	2.61 ± 0.26§	1.65 ± 0.27*	2.71± 0.35§
Vitamin E (mg.dl^{-1})	1.82 ± 0.09	0.78 ± 0.07*	1.97 ± 0.10§	0.85 ± 0.04*	1.68 ± 0.08§

The values expressed as mean ± SEM (n=6). All group vs. control group at 11-weeks, * P<0.05; and all group *vs.* Pb group at 11-weeks, § P<0.05 (Student's ' *t* ' test).

all groups treated (Pb/Abs and Water/Abs groups) we noticed a significant difference compared to water group by +19.7% and 37.2%, respectively (p<0.05).

Table 2 shows the concentrations of vitamins E, C and NP-SH in plasma, GSH in RBC of all groups. We observed a reduction in GSH concentrations (-28.9%) in Pb compared to the concentrations found in water group after 11-week of intoxication; but no significant difference was observed between water/Abs and Pb/Abs groups compared to control group. However, we noticed respectively a relative -32.9% and -28.5% decreased concentration in vitamin C in Pb and Pb/water groups compared to water group; the concentration of vitamin E was significantly diminished in Pb and Pb/water group compared to control group by -57.1% and -53.3%, respectively, no difference was noted between Pb/Abs and control group. We noticed a significant increased value in NP-SH of Pb, Pb/Water and Pb/Abs groups compared to control group (-53%, -38.9% and-14.2%, respectively).

Figure 2 gives the activities of enzymatic antioxidants in the erythrocytes hemolysate of all groups. Rats intoxicated with lead acetate (Pb group) showed significant reduction in the activities of GR compared to control group after 11-week of intoxication (-33.8%); the activity of SOD and GPx was significativelly increased by about +64.3% and +40.3% compared to control group. After a 4-week treatment, the activity of GPx was significantly reduced compared to control group (-15%), and the SOD activity was significantly increased by +16.4% compared to control group. No difference was noted between all groups in the catalase activity.

Discussion

Our previous investigations showed that chronic treatment with Pb induced oxidative damage in erythrocytes of rats, causing destruction of cell membranes and increased lipid peroxidation, as well as alteration of the antioxidant defence system, energy metabolism and the appearance of anaemia (Gurer et al., 1998; Hiraku and Kawanishi, 1996; Costa et al., 1997; Sugawara et al., 1991).

The results obtained in our present study show that treatment with Pb induces an increased LP concentration in the blood of rats (Figure 1), which was accompanied by increased formation of ROS (Gurer and Ercal, 2000). As a consequence, enhanced lipid peroxidation, DNA damage, altered calcium and sulfhydryl homeostasis as well as marked disturbances of antioxidant defence system occurred (Hiraku and Kawanishi, 1996). Treatment with wormwood extract was very effective in decreasing the oxidative damage induced by Pb which resulted in significantly lower LP concentration. The wormwood extract was capable of inhibiting formation, and antagonizing H_2O_2 which caused haemolysis, indicating that these plant extracts are scavengers against hydroxyl radicals.

In animals exposed to Pb, the activities of SOD and GSH-Px were significantly increased (Figure 2). It is known that Pb induces the formation of superoxide anion radicals in erythrocytes and it is reasonable to expect an increased activity of SOD (Costa et al., 1997, Ye et al., 1999). The mechanism of Hb oxidation to metHb and superoxide, followed by SOD catalyzed dismutation of superoxide to H_2O_2, has long been known; and it is proposed that the high metHb values found in the lead-exposed workers reflect both lead-induced direct oxyHb oxidation and co-oxidation of oxyHb with ALA (Monteiro et al., 1986).

Pb induced an increase in CAT and GSH-Px activities which may be explained by their influence on hydrogen peroxide as substrate which is formed in the process of dismutation of superoxide anion radicals (Chiba et al., 1996). Lead also binds to enzymes that have functional sulfhydryl groups, rendering them non-functional and further contributing to impairment in oxidative balance. Levels of two specific sulfhydryl containing enzymes that are inhibited by lead (ALAD and GR) have been demonstrated to be depressed in both animal and human lead-exposure studies (Ahamed et al., 2005; Gurer-Orhan et al., 2004).

The treatment with wormwood extract after Pb administration decreased erythrocyte SOD, CAT and GPx activities indicating that this extract eliminates the toxic effects of Pb on the activity of these enzymes. At the same time, RBC reduced GSH concentrations remain at the level of control values which confirm the protective role of wormwood extract.

GR, the enzyme responsible for recycling of glutathione from the oxidized form (glutathione disulfide; GSSG) to the reduced form (reduced glutathione; GSH) is also deactivated by lead (Hunaiti et al., 1995). Depressed levels of glutathione reductase, glutathione peroxidase, and glutathione-S-transferase were all found to correlate with depressed glutathione levels in occupationally-exposed workers (Sandhir and Gill, 1995). The antioxidant treatment after Pb administration helped to maintain the erythrocyte GSH content (Flora et al., 2004; Flora et al., 2003).

As is shown in Table 2, a significantly decreased Vitamin C and Vitamin E concentrations in the plasma of rats after acute Pb treatment were observed. Other investigations showed that chronic treatment with Pb induces a decrease of Vitamin C concentration in plasma, liver and kidney; Vitamin C is a known free-radical scavenger and has been shown to inhibit lipid peroxidation in liver and brain tissue of lead-exposed animals (Padh, 1991). Vitamin C also protects plasma lipids against lipid peroxidation and has an important role in the regeneration of α-tocopherol (Patra et al., 2001).

Vitamin C is a potent scavenger of free oxygen radicals and it has been shown that marginal Vitamin C deficiency results in intracellular oxidative damage in the guinea-pig (Tatara and Ginter, 1994); And lead can accelerate the oxidation of vitamin C, causing it is to lose its bioactivity. In comparison to the chronic exposure, the acute treatment demonstrated that increased concentrations of Vitamin C and Vitamin E may be due to a defence response of the organism to oxidant injuries caused by Pb.

ALAD is a crucial enzyme in lead toxicity because the inhibition of ALAD lowers haeme production and increases levels of the substrate ALA in blood and urine (Table 1). Elevated levels of ALA found both in the blood and urine of subjects with lead exposure, are known to stimulate ROS production (Bechara, 1996). Together, with our results showing oxidative stress associated with increased of urinate ALA; the current data provide strong evidence for the contribution of ALA to the prooxidant effects and toxicity of lead in all animals treated by lead acetate. In addition, we observed that urine concentrations of ALA provide a useful index of the physiological response to lead exposure.

Conclusion

It can be concluded from presented results that lead induced oxidative damage in erythrocytes leads to loss of membrane function by enhancing LP as well as alteration of the activity of AOS enzymes and concentrations of reduced GSH, Vitamin C, E and NP-SH. Naturally occurring antioxidants have been extensively studied for their capacity to protect organisms and cells from oxidative damage. The aqueous extract of Wormwood (*Artemisia absinthium*) expressed protective role against toxic influence of lead on all analysed parameters in rat.

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